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RESEARCH ARTICLE

Validation of Functional Markers Associated with Genes for Fragrance in Rice (*Oryza sativa* L.)

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The presence of aroma determines the market price of rice and is associated with its local, cultural and national identity. The present study was carried out to validate five fragrance linked functional markers and to evaluate the fragrance locus based molecular diversity among the genotypes of rice. The experimental material consists of 24 genotypes of rice including both aromatic and non-aromatic indica and japonica types. None of the markers was found to differentiate all the fragrant genotypes from the non-fragrant ones with 100% efficacy. The markers could not differentiate fragrant genotypes like Mushk Budgi, Kamad, Kalanamak and Jeera Battis, from non-fragrant ones. Moreover, these fragrant genotypes were clustered with non-fragrant ones. This supports the existence of some other gene responsible for fragrance other than *badh2*, thereby indicating that aroma in rice is being governed by two or more genes. This also reveals the scope for development of more markers through fine mapping of large number of genotypes or through association mapping for differentiation of aromatic and non-aromatic rice.

Key Words: *Badh2*, 2AP, Fragrance, Functional markers, Rice

Introduction

Rice serves as the main staple food for half of the world population and two-third of Indians depend on rice for their survival. The quality considerations in this crop assume enhanced importance, especially in the countries which are self-sufficient in their production. As living conditions are steadily improving, human demand for high quality rice is continuously on increase. This demands in incorporation of preferred grain quality features as the most important objective next only to yield enhancement (IIRR, 2015). Among the various quality traits in rice, the presence of aroma determines its market price and is associated with local, cultural and national identity (Kovach *et al.,* 2009). Jasmine and basmati rices with distinct aroma are highly preferred by consumers throughout the globe.

Aroma is a highly complex trait and more than one hundred volatile aromatic metabolites are responsible for its expression. However, only a handful of these metabolites are considered to influence rice aroma significantly (Mathure *et al.,* 2014). Among these, 2-acetyl-1-pyrroline (2AP) is considered to be the principal component associated with a popcorn-like aroma in rice (Lorieux *et al.,* 1996). 2AP has a very low odour threshold and hence can influence the aroma phenotype and be detected by consumers at very low concentrations (Nadaf *et al.,* 2006). The compound is produced through a single recessive allele (*fgr*) at a locus on chromosome 8 (Lorieux *et al.,* 1996; Chen *et al.,* 2006), which corresponds to the gene that encodes betaine aldehyde dehydrogenase (*BADH2*). An 8-bp deletion in exon 7, introducing a premature stop codon upstream of key coding regions, makes this gene nonfunctional (*badh2*), and consequently the mutant *badh2* transcript leads to 2AP accumulation in aromatic rice (Hashemi *et al.,* 2013). Based on this locus, several PCR-based markers have been developed making its detection inexpensive, simple and rapid. However, most of the markers identified are located physically away from the gene and therefore may or may not be efficient in marker-assisted selection (MAS) for aroma. Although some functional markers have also been developed based on sequence variations within the *fgr* gene (Amarawathi *et al.,* 2008; Shi *et al.,* 2008; Sakthivel *et al.,* 2009), their evaluation in Indian aromatic germplasm has not been done so far. Moreover, these markers have been

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Table 1. Genotypes used in the present study.

S. No.	Genotypes	Origin	Fragrance	Remarks
1.	Jaya	Punjab	None	Released variety (Indica)
2.	NDR-359	Uttar Pradesh	None	Released variety (Indica)
3.	Sarjoo-52	Uttar Pradesh	None	Released variety (Indica)
4.	Basmati-370	Punjab	Strong	Traditional basmati (Indica)
5.	Taraori Basmati	Haryana	Strong	Traditional basmati (Indica)
6.	Type-3	Uttarakhand	Strong	Traditional basmati (Indica)
7.	Dehradun Basmati-1	Uttarakhand	Strong	Traditional basmati (Indica)
8.	Dehradun Basmati-2	Uttarakhand	Strong	Evolved basmati (Indica)
9.	Mushk Budgi	Jammu & Kashmir	Moderate	Landrace (Japonica)
10.	Kamad	Jammu & Kashmir	Moderate	Landrace (Japonica)
11.	Quadir Beigh	Jammu & Kashmir	None	Landrace (Japonica)
12.	Mehvan	Jammu & Kashmir	None	Landrace (Japonica)
13.	Begum	Jammu & Kashmir	None	Landrace (Japonica)
14.	Mazha	Jammu & Kashmir	None	Landrace (Japonica)
15.	Adam Chini	Uttar Pradesh	Strong	Landrace (Indica)
16.	Badshah Bhog	Assam	Strong	Landrace (Indica)
17.	Juhi Bengal	West Bengal	Strong	Landrace (Indica)
18.	Kalanamak	Uttar Pradesh	Strong	Landrace (Indica)
19.	Jeera Battis	Uttar Pradesh	Strong	Landrace (Indica)
20.	Tulsi Manjari	Bihar	Strong	Landrace (Indica)
21.	Hariram-48	Uttar Pradesh	Strong	Landrace (Indica)
22.	Dubraj	Madhya Pradesh	Strong	Landrace (Indica)
23.	Sonachur	Uttar Pradesh	Moderate	Landrace (Indica)
24.	Laung Choor	Uttar Pradesh	Moderate	Landrace (Indica)

developed for basmati and/or jasmine rice and their usefulness in short and medium-grained indigenous landraces have not been tested yet. The present study was carried out to validate some reported functional markers in a set of aromatic and non-aromatic rice genotypes. The level of fragrance locus based genetic diversity within this set of rice genotypes was also evaluated on the basis of these functional markers.

Materials and Methods

The study was conducted during 2015 at Central Laboratory Facility of the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi (UP). The site of study is situated at 25°18´N latitude and 83°03´E longitude at an elevation of 80.71 m above mean sea level. The experimental material consists of 24 genotypes of rice including aromatic, non-aromatic and temperate japonicas (Table 1). For molecular analysis, 5 reported (Rai *et al.,* 2015) fragrance related functional markers associated with linkage group 8 were used for the given set of genotypes (Table 2).

The young leaves were collected from 7-10 days old seedlings and immediately stored at -20°C till further processing. DNA extraction from these leaf samples were carried out following the CTAB extraction method (Doyle and Doyle, 1987) with few modifications. DNA quality was evaluated by electrophoresis in 0.8% agarose gel and quantification was accomplished using spectrophotometer. DNA amplification was carried out in 10 µl reaction mixtures containing 5 ng/10µl template DNA, 1×PCR buffer, 2.4 mM MgCl2, 0.12 mM dNTPs, 0.7 pM of each primer (forward and reverse) and 0.6 U of

Table 2. Allele size (bp) and polymorphism information content (PIC) of the functional markers used in the present study.

S. No.	Primer name	No. of alleles	Allele size (bp)	PIC
1.	ESP+IFAP+INSP+EAP	3	257/355/580	0.49
2.	CP04133	2	421/483	0.75
3.	nksbad2	2	82/90	0.75
4.	FMbadh2-E7	2	260/270	0.75
5.	BADEX7-5	2	95/108	0.75
6.	Total alleles	11	Average PIC	0.69

Taq DNA Polymerase. Polymerase chain reaction (PCR) was carried out in a thermal cycler (Eppendorf, USA) with the temperature cycle profile: Initial denaturation at 94°C for 4 min, 40 cycles each of 1 min denaturation at 94°C followed 30 sec annealing at 55°C to 65°C (depending on the primer used) and 1 min extension at 72°C, and finally 4 min at 72°C for the final extension. The amplified products were separated in 2.5 percent agarose gel prepared in 1×TAE (Tris-acetate-EDTA) buffer and stained with ethidium bromide. The gels were run in 1x TAE buffer at constant voltage of 65 V for 3 hours.

They were visualized and photographs taken using gel documentation instrument (BioRad). Clearly resolved and unambiguous bands for each primer were scored in the form of matrix as 1 (presence) and 0 (absence) for each genotype. The binary data matrix was then utilized to generate genetic similarity data among the genotypes.

The binary data matrix generated by functional markers was subjected to further analysis using NTSYS-pc version 2.11W (Rohlf, 1997). The SIMQUAL program was used to calculate the Jaccard's similarity coefficients. The similarity matrix was used as an input for generating the clusters. Unweighted Pair Group Method based on Arithmetic Average (UPGMA) clustering was done using SAHN module of NTSYSpc for dendrogram construction. Polymorphic information content (PIC) was estimated using the formula proposed by Nei (1973).

$$PIC_i = 1 - \Sigma P_{ij}^2$$

Where PIC*i* is the polymorphic information content of a marker *i*, P*ij* is the frequency of the j^{th} pattern for marker *i* and the summation extends over *n* patterns.

Results

The study was intended to validate five known functional markers and to evaluate the fragrance locus based molecular diversity among the genotypes of rice. All the five primers used were found to be polymorphic (Table 2) and yielded a total of 11 fragments (amplified products). The size of fragments varied from 82 bp (by marker nksbad2) to 580 bp (by allele specific primer ESP+IFAP+INSP+EAP). Maximum of three alleles were amplified by marker ESP+IFAP+INSP+EAP, while rest of the markers yielded two alleles each.

Allele specific amplification by marker ESP+IFAP+INSP+EAP generated three alleles of 257, 355 and 580 bp size. The largest fragment of 580 bp was amplified in all the genotypes. In addition, a second fragment of either 257 or 355 bp was amplified in the genotypes using this primer. In all the fragrant genotypes except, Mushk Budgi, Kamad, Kalanamak and Jeera Battis, a 355 bp fragment was amplified. The exceptional genotypes along with the non-fragrant ones amplified a 257 bp fragment, in addition to 580 bp fragment (Fig. 1). Marker CP04133 generated a 483 bp fragment in all the fragrant genotypes except Mushk Budgi, Kamad, Kalanamak and Jeera Battis. A 421 bp fragment was produced in these exceptional and in non-fragrant genotypes (Fig. 2). Marker nksbad2 generated an 82 bp fragment in all the fragrant genotypes except for Mushk Budgi, Kamad, Kalanamak and Jeera Battis. While a 90 bp fragment was produced in these exceptional genotypes and in non-fragrant ones. Marker FMbadh2-E7 generated a 260 bp fragment in all the fragrant genotypes with the exception of Mushk Budgi, Kamad, Kalanamak and Jeera Battis. While a 270 bp fragment was produced in these exceptional and in non-fragrant genotypes. Marker BADEX7-5 generated a 95 bp fragment in all the fragrant genotypes except for Mushk Budgi, Kamad, Kalanamak and Jeera Battis. These exceptional genotypes along with the non-fragrant lines produced a 103 bp fragment. Highest polymorphism information content (PIC) value of 0.75 was revealed by the primers CP04133, nksbad2, BADEX 7-5 and FM badh2-E7, while lowest PIC value of 0.49 was shown by the primer ESP+IFAP+INSP+EAP (Table 2).

The genetic similarity coefficient obtained in the present study ranged from 0.09 to 1.00 due to diversification in the genotypes at the fragrance locus. All the fragrant genotypes except Mushk Budgi, Kamad, Kalanamak and Jeera Battis, revealed the similarity coefficient of 1.00 among themselves. The non-fragrant genotypes along with Mushk Budgi, Kamad, Kalanamak and Jeera Battis, also revealed the similarity coefficient of 1.00 among themselves. The genetic similarity among the two groups (clusters) was found to be 0.09. A dendrogram based on Jaccard's similarity coefficients and constructed using UPGMA is shown in Fig. 3. In the dendrogram, 24 rice genotypes were grouped into two clusters. Clusters I and II consisted of 11 and 13 genotypes, respectively. Cluster I includes Jaya, NDR-359, Sarjoo-52, Mushk Budgi, Kamad, Quadir Beigh, Mehvan, Begum, Mazha, Kalanamak and Jeera Battis. On the other hand, cluster II includes Basmati-370,

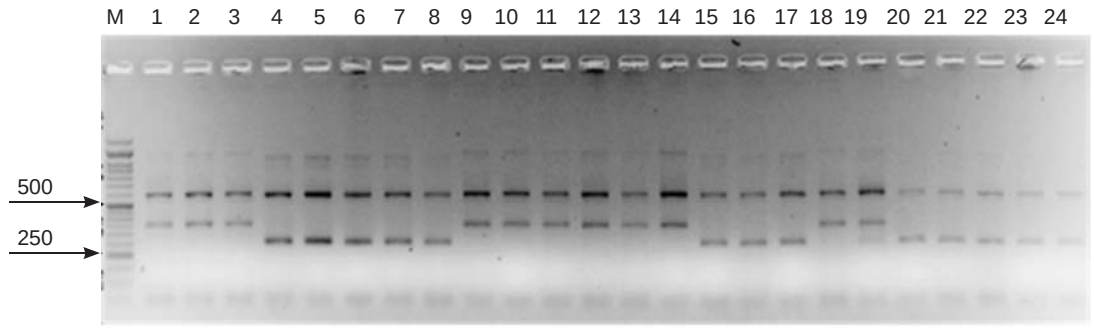

Fig. 1. Banding patterns generated by a functional marker ESP+IFAP+INSP+EAP. The lane number corresponds to the genotypes shown in Table 1.

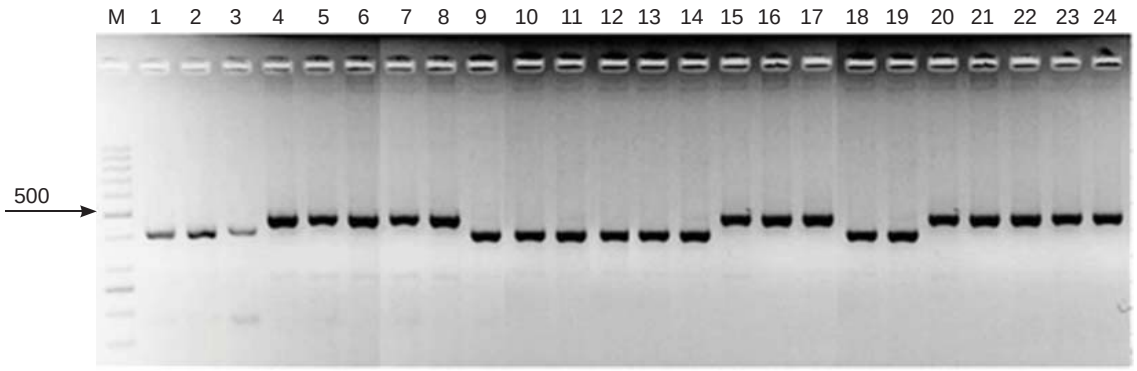

Fig. 2. Banding patterns generated by a functional marker CP04133. The lane number corresponds to the genotype shown in Table 1

Taraori Basmati, Type-3, Dehradun Basmati-1, Dehradun Basmati-2, Adam Chini, Badshah Bhog, Juhi Bengal, Tulsi Manjari, Hariram-48, Dubraj, Sonachur and Laung Choor.

Discussion

Identification of molecular markers related to grain and cooking quality features has been a matter of great interest since last several decades. The molecular markers serve several functions, including marker-assisted selection (Singh *et al.,* 2011), identification of regions affecting quantitative trait loci (Collard *et al.,* 2005) and estimation of genetic diversity (Myint *et al.,* 2012; Rai *et al.,* 2015). The practical application of MAS requires the development of tightly linked, cost effective and easy to use molecular markers. The present study illustrates the utility of molecular markers to identify the presence of *fgr* gene in rice and to establish genetic relationships among various fragrant and non-fragrant genotypes. In this study, five functional markers were evaluated for their efficacy to discriminate the fragrant and non-fragrant genotypes. Since the marker's importance is based on its proximity to a gene of interest, gene-derived functional markers are inherently more valuable than microsatellite or random sequence-based markers.

In the present study, none of the markers could differentiate all the fragrant genotypes from the non-fragrant ones with 100% efficacy. The markers could differentiate all the fragrant genotypes, except Mushk Budgi, Kamad, Kalanamak and Jeera Battis, from non-fragrant ones. Therefore, these markers can be used for identification, discrimination and MAS for aroma in traditional basmati, evolved basmati and local aromatic landraces, excluding Mushk Budgi, Kamad, Kalanamak and Jeera Battis. Mushk Budgi and Kamad are the japonica type fragrant genotypes and may have different gene for fragrance other than *badh2* locus. Rai *et al.* (2015) reported similar results while evaluating five functional markers in a set of 24 genotypes. Their studies revealed that none of the markers could differentiate fragrant and non-fragrant genotypes with 100% efficacy. This supports the hypothesis of a second gene for fragrance (Fitzgerald *et al.,* 2008) in some non-basmati aromatic genotypes (such as Kalanamak, Jeera Battis) and in aromatic japonicas (such as Mushk Budgi, Kamad).

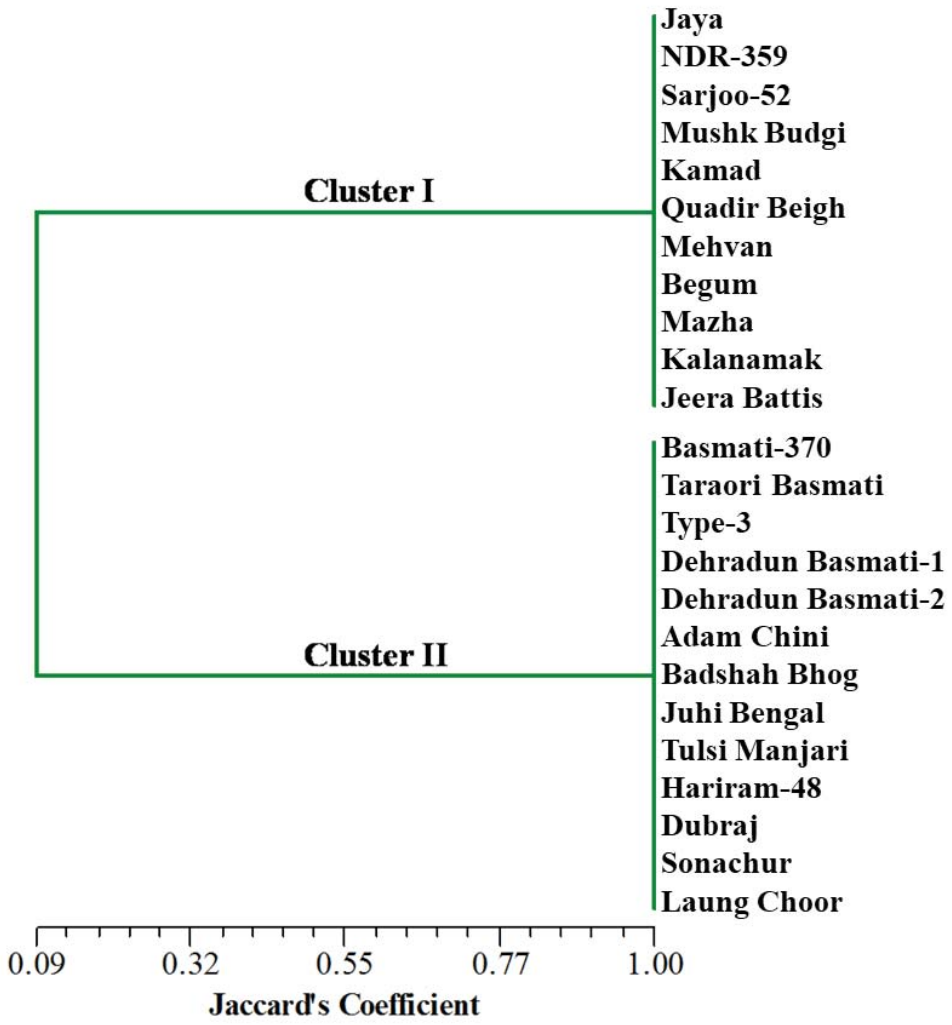

Fig. 3. UPGMA based dendrogram of 24 rice genotypes, generated by the data of five functional markers

The genetic diversity of Indian fragrant rice varieties is expected to be high due to its rich ecological diversity. In the present study, all the genotypes were grouped into two clusters corresponding to fragrant and non-fragrant clusters with few exceptions. All the non-fragrant genotypes along with Mushk Budgi, Kamad, Kalanamak and Jeera Battis were grouped into one cluster. The basmati types along with other short and medium grain fragrant genotypes were readily separated from the non-fragrant cluster. Among basmati type fragrant genotypes, all markers were monomorphic, indicating that they share a common gene for fragrance. The fragrant rices, Kalanamak and Jeera Battis, were separated distantly from all the medium-grained aromatic genotypes, most likely due to the presence of different gene for aroma. The locus based inter-cluster distance was very high and no difference was observed among genotypes of the same cluster. This may be due to the reason that the present study was based on a single locus and less number of markers were used for analysis.

The studies on diversity of *BADH2* gene in a large collection of accessions have revealed that an 8-bp deletion in the seventh exon is present in most of the aromatic accessions, but other less frequent mutations associated with fragrance have also been detected (Shi *et al.,* 2008; Kovach *et al.,* 2009; Myint *et al.,* 2012). Additionally, several aromatic accessions do not carry any mutation in their coding segments (Singh *et al.,* 2010; Myint *et al.,* 2012) and the reason for their fragrance is unknown. Fitzgerald *et al.* (2008) postulated that the production of 2AP is being driven by alleles of 2 different genes besides the different alleles of a

BADH2 gene. Thus, rice has indeed a second *BADH* enzyme, which acts in a similar way as that of *BADH2*, but is regulated differently. Furthermore, Mushk Budgi, Kamad, Kalanamak and Jeera Battis were clustered with non-fragrant genotypes, although they possess fragrance. This also indicates the existence of some other gene responsible for fragrance other than *badh2*. Annotation of the rice genome database between *aro4-1* QTL intervals indicate the presence of a gene for *BADH1*, which could be a likely candidate gene for aroma, due to its similar molecular function as that of *badh2* (Singh *et al.,* 2010). This further reinforces the speculation that the fragrance is being governed by two or more genes. This reveals the scope for development of more markers through fine mapping of large number of genotypes or through association mapping for differentiation of aromatic and non-aromatic rice.

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